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Review

Some applications of thermophiles and their enzymes for protein processing

Józef Synowiecki

Department of Food Chemistry, Technology and Biotechnology, Faculty of Chemistry, Gdansk University of Technology, ul. Gabriela Narutowicza 11/12, 80-233 Gdańsk, Poland. E-mail: synowiec@chem.pg.gda.pl. Tel: (48-58) 347-1395. Fax: (48-58) 2694.

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Proteolytic enzymes produced by thermophiles are of considerable interest because they are stable and active at elevated temperatures. Moreover, they are resistant to organic solvents, detergents, low and high pH and other denaturants. Such properties allow many technological processes. Advantages of thermozymes applications are reduced risk of microbial contamination, increased mass transfer, lower viscosity and improved susceptibility of some proteins to enzyme molecules. This review covers thermostabilization strategies and some properties of thermostable proteases as well as their current and future applications in food processing, medicine and some other industries.

Key words: Thermozymes, keratinases, proteases, thermophiles.

INTRODUCTION

Peptidases commonly designated as proteases (EC 3.4) are important group of enzymes produced mainly for food, pharmaceutical, detergent, leather and textile industries. In food processing, proteases are used extensively for the modification and improvement of protein functionality, production of protein hydrolyzates, meat tenderization, utilization of different by-products, as well as for catalysis of the plastein reactions (Adler-Nissen, 1986; Aspamo et al., 2005; Blackebrough and Birch, 1981). A limited hydrolysis is advantageous to improve protein functional properties, such as solubility, emulsifying capacity and foaming and gel forming ability. On the contrary, deep hydrolysis leads to products containing short peptides and free amino acids (Lahl and Braun, 1994; Moure et al., 2005). In many cases, selective proteases are necessary, e.g., for the development of desirable flavor, removing bitter peptides or for production of bioactive compounds (Raksakulthai and Haard, 2003; Synowiecki, 2008; Uhlig, 1998).

Proteases are highly diverse enzymes having different active sites. They are classified into exopeptidases, that attack the ends of protein molecules, and endopeptidases which cleave peptide bonds within polypeptide chains. Depending on the nature of the functional group at the active site they are divided into serine proteases, aspartic proteases, metalloproteases, cysteine proteases and endopeptidases of unknown catalytic mechanism (Grzonka et al., 2007; Mansfeld, 2007; Rao et al., 1998).

Cleavage of peptide bonds can not occur when they are placed in the interior of the protein structure and are not accessible to enzyme without unfolding, e.g., after denaturation. Furthermore, the effectiveness of different proteases depends on kind of amino acids that generate the peptide bonds. Nonspecific enzymes can hydrolyze a large number of peptide bonds, however, they do not break them at the same rate (Rao et al., 1998). Thus, the enzyme specificity should be kept in mind when considering an appropriate protease for use in a particular process. Possible proteases applications are also influenced by the composition of catalytic site. Cysteine proteases are susceptible to inhibition by several reagents, particularly oxidants and metal ions. This is the reason why most of the thermophiles synthesize serine- or neutral metalloproteases. However, metalloproteases are rapidly inactivated by the removal of metal ions and can not be used in reaction media containing chelating agents (Mansfeld, 2007).

Thermostable proteases like other enzymes from thermophilic and hyperthermophilic microorganisms (often called thermozymes) are of particular interest in some applications because they are stable and active at temperatures above 60 - 70°C. Furthermore, they are

more resistant than their mesophilic counterparts to organic solvents, detergents, low and high pH and other denaturing agents (Covan et al., 1985; Covan, 1997; Gupta and Khare, 2006). The use of proteases at higher temperatures is beneficial, because unfolded form of proteinaceous substrate is better susceptible for the still active thermostable protease. It results in higher specific activities for proteases sourced from thermophiles and optimization of some industrial processes, particularly with the enzymes that are active at temperatures near 100°C. The resistance against organic solvents makes the thermostable proteases useful for synthesis of high-molecular weight peptides, carried out in reaction media with low water content (Bruins et al., 2001; Sellek and Chaudhuri, 1998; Synowiecki, 2008). Performing enzyme reactions at elevated temperatures allow for higher substrate concentrations, lower viscosity, reduction of microbial contamination risk and high reaction rates (Bruins et al., 2001; Eichler, 2001).

Thermozymes can be purified easily by heat treatment when expressed in mesophilic hosts. This way of purify-cation of the recombinant thermostable enzymes is quite satisfactory for many industrial applications. Additional benefit of some thermostable enzymes is decreased activity at low temperatures and this feature enables termination of the reaction just by cooling (Vieille and Zeikus, 2001). Thus the use of inhibitors or expensive enzyme removing procedures after completion of the reaction can be avoided. Main disadvantages of thermo-zymes application are the increased possibility of by-products formation and the degradation of thermolabile substrates or products. Currently, many heat resistant proteases have been studied but most of them are still not available in the market.

THERMOSTABILIZATION STRATEGIES

Thermal resistance of enzymes is determined by free-energy consumption necessary for transformation of molecules from folded to unfolded state (Shiraki et al., 2001). Slight changes of amino acids distribution and sequences increase the number of stabilizing interactions in the folded protein, such as: additional ion-pairs, disulphide bridges, hydrogen bonds and hydrophobic interactions (Farias and Bonato, 2003; Kumar and Nussinov, 2001; Mozhaev, 1993). In addition to side-chain interactions, thermophilic and hyperthermophilic microorganisms adopt other strategies for stabilizing proteins. This may be achieved by filling cavities in the molecular structure of the proteins, shortening of the loops and reduction of accessible hydrophilic surface area (Fukuchi and Nishikawa, 2001; Shiraki et al., 2001; Stetter, 1999; Thompson and Eisenberg, 1999; Voght et al., 1997). Other modifications include: metal ion binding and diminished amount of residues susceptible to deamidation or oxidation. Moreover, some thermozymes contain termolabile residues in location in which they are not susceptible to degradation (Zeikus et al., 1998).

Thermostability of some enzymes can be assured by environmental factors, e.g., increased intracellular salts and protein concentrations and synthesis of different stabilizers (Gupta, 1991; Vieille and Zeikus, 2001). However, there are no universal factors or their combinations that may be responsible for thermal stability of proteins. Furthermore, many features involved in thermo-stabilization of soluble proteins do not appear in case of membrane proteins (Schneider et al., 2002; Trivedi et al., 2006). Although, adaptation of thermoz-ymes to act at elevated temperatures is mainly achieved by exchange of few amino acid residues and/or their different localization in molecule, the homologous ther-mostable and thermo-labile enzymes are similar and have the same catalytic mechanisms (Vieille and Zeikus, 2001). Higher resistance of thermozymes as compared with their mesophilic counterparts is the result of increased rigidity, which preserves their catalytically active structure, but leads to reduced activity at lower temperatures (Shiraki et al., 2001). However, in some cases, thermozymes are more active than their mesophilic counterparts even at low temperatures. Such phenomenon suggests that their molecules exhibit local flexibility in the area of catalytic site with overall rigidity of the rest of the protein (Vieille and Zeikus, 2001). It is interesting to note that most of the heat resistant enzymes indicate maximal activity above the optimal growth temperature of the microorganisms from which they are isolated (Fujiwara, 2002; Niehaus et al., 1999).

SOME SOURCES OF THERMOSTABLE PROTEASES

Proteases are physiologically important molecules and their synthesis is widespread in plants, animals and different strains of bacteria, fungi and yeasts. An excellent source of proteases are microorganisms, and among them the great attention received thermophilic bacteria and archaea which are classified into moderate- or extreme thermophiles and hyperthermophiles, growing optimally at 50 - 60°C, 60 - 80°C or 80 - 113°C, respectively (Fujiwara, 2002; Lasa and Berenguer, 1993). Thermophilic microorganisms were found in different biotopes, such as hot springs, geothermal sediments, marine solfatares or fermenting compost as well as in industrial environments, e.g., hot water pipelines (Rothschild and Manicineli, 2001). The majority of hyperthermophiles belong to the archaea growing usually at anaerobic biotopes (Antranikian et al., 1995).

Unconventional growth conditions of many hyperther-mophiles, relatively low cell and enzyme yields, and sometimes generation of toxic and corrosive metabolites cause technological difficulties during large-scale enzyme production (Danson et al., 1992; Huber and Stetter, 1998). Moreover, the cultivation conditions and composi-

tion of the growth media controlling protease production in hyperthermophiles are still not enough recognized. These problems can be eliminated by expression of the genes responsible for synthesis of the desired enzyme in mesophilic host. In some cases, decomposition of proteinaceous by-products is achieved during cultivation of microorganisms with strong proteolytic activity. Unfortunately, such mesophiles can often invade the human and animal tissues which cause their pathogenity (Suzuki et al., 2006). However, there have been no reports of the pathogenity of obligate thermophiles. Thus, thermophiles have a great advantage in terms of their safe use.

Thermophilic and hyperthermophilic microorganisms studied during the recent years fulfill demand on different proteolytic enzymes that have optimum pH up to 12.0 and temperatures ranged from 45 - 110°C (Antranikian et al., 1995). At present, industrially important thermostable proteases are usually produced using thermophilic strains belonging to the genus *Bacillus* (Haaki and Rakshit, 2003). An example of such enzyme is thermolysin, a neutral metalloprotease isolated from *Bacillus stearothermophilus* with half-life of 1 h at 80°C (Rahman et al., 1994; Rao et al., 1998). The pyrolysin, serine protease which reach maximal activity at 100°C was isolated from hyperthermophilic archaeon *Pyrococcus furiosus* (Antranikian et al., 1995). Other examples of well characterized heat resistant proteases are subtilisins of *Bacillus* origin. They exhibit broad substrate specificity and have similar properties such as optimal temperature of 60°C and an optimal pH of 10 (Klingberg et al., 1991; Rao et al., 1998). A valuable source of thermostable proteases seems also to be gram-negative, aerobic bacteria of the genus *Thermus* which have been isolated from many natural and artificial thermal environments. The strains belonging to the genus *Thermus* utilize carbohydrates, amino acids, carboxylic acids and peptides and their optimal growth temperatures ranged from 55 - 85°C. High culture yields make this microorganism valuable source of different thermozymes. Some *Thermus* strains, e.g., *Thermus aquaticus* and *Thermus thermophilus* can be used as a source of aqualysin which is subtilisin type heat-stable serine protease. This enzyme is secreted as zymogen activated in the growth medium through autolysis (Pantazaki et al., 2002). Other proteolytic enzyme sourced from *Thermus* strains is ATP dependent zinc protease which catalyzes release of small peptides (Pantazaki et al., 2002).

A number of proteases have been isolated from archaeons (Klingberg et al., 1991; Hanazawa et al., 1996). Among them *Desulfurococcus kamchatkensis* is able to grow at temperatures between 65 and 87°C and can hydrolyze wide range of substrates, including α-keratin, albumin or gelatin (Kublanov et al., 2009). Thermophilic microorganisms exhibiting keratinolytic properties have a great significance because they can be used for decomposition of waste by-products from poultry industry which are generated in amount about 10,000 tons annually (Suzuki et al., 2006).

Unfortunately, the properties of thermostable keratinases have been reported only in a few cases. Among them keratinase isolated from *Fervidobacterium islandicum* AW-1 was characterized (Nam et al., 2002). In some cases thermostable keratinases active at temperatures about 70°C are produced by mesophiles (Dozi et al., 1994; Takami et al., 1992).

Enzymes active towards collagen are useful for utilization of bones and some other wastes from meat industry. However, most mesophiles producing collagenases have ability to invade human tissues by injuring extracellular matrix and this limits the application of such pathogens (Watanabe, 2004). On the other hand, there are rare pathogenic strains of thermophiles, even among those that decompose collagen. Unfortunately, collagenases are seldom identified in thermophilic sources. Recently, collagenolytic activity of *Geobacillus collagenovorans*, growing at temperatures 50 - 70°C in a neutral pH range was reported (Miytake et al., 2005). Another example of thermophile active against collagen (reported in the article of Okamoto et al., 2001) is *Alicyclobacillus sendainensis* NTAP-1 which preferentially grows in acidic environment.

COMMERCIAL USE AND FUTURE PROSPECTS

Currently, only few thermostable proteases are comercially available. One of them is alcalase isolated from *Bacillus licheniformis*. The major ingredient of this preparation is subtilisin, which is an endoprotease of serine type, exhibiting highest activity at 60°C and pH of 8.3. Alcalase found many applications in the food industry, e.g., by reason of their low specificity towards different proteins from plant and animal sources. For example, this enzyme is important in the processing of soy meal which results in soluble, non-bitter hydrolyzate, used as component of protein-fortified soft drinks and dietetic food (Synowiecki, 2008). Alcalase is also useful for recovery of proteins from by-products of the meat and fish industry and from crustacean shell waste during chitin production. Furthermore, thermostable proteases that are resistant to anionic or non-ionic surfactants and are active at temperatures above 60°C found application as component of dishwashing detergents (Banerjee et al., 1999; Niehaus et al., 1999). Such enzymes can be also used for cleaning ultrafiltration membranes at high temperatures, increasing the efficiency of this process (Bruins et al., 2001).

The other potential application of heat-resistant proteases is meat tenderizing. It is due to the great difference of enzymatic activity at moderate and high temperatures. The mesophilic proteases injected into the tissue show a residual activity during the whole period of post-slaughter storage of the meat cuts leading to an

excessive fragmentation of the protein molecules.

The use of enzymes with an essential activity only during cooking allows the stoppage of proteolysis just by cooling. The stability of heat-resistant proteases in aqueous/organic and nonaqueous media leads to the modification of the reaction equilibria, creating new peptide bonds. Such reverse reactions may be used to improve nutritional quality and functionality of protein hydrolyzates and to reduce their bitterness. It is achieved by increased molecular weight of peptides as well as by introduction of desirable amino acid residues. Among the thermostable proteases currently used on an industrial scale, immobilized thermolysin from *Bacillus thermoproteolyticus* is involved in the synthesis of aspartyl-phenylalanine-1-methyl ester, known as aspartame (De Martin et al., 2001). This product is commonly used as sweetener in many low-caloric food and beverages. Enzymatic synthesis eliminates contamination of the product by non-sweet and bitter isomers.

Poultry industry generates a large amount of feathers. Traditionally, this by-product is degraded by alkali hydrolysis and steam pressure cooking. Such way of processing destroy some essential amino acids and lead to formation of non-nutritive lysinoalanine and lantionine. By this reason, a great significance has non-polluting, biotechnological utilization of keratin-containing wastes (Gousterova et al., 2005; Grazziotin et al., 2006). However, the commonly produced proteases could not degrade keratin. It is caused by tightly packed filament structure of keratin, stabilized by a large number of disulfide- and hydrogen bonds as well as by hydrophobic interactions (Parry and North, 1998). This insoluble protein can be broken down by some microorganisms secreting keratinases (EC 3.4.99.11) and obtained hydrolyzates have been used as fertilizers and dietary protein supplement for animal feed (Grazziotin et al., 2006). In addition, keratinases have potential use as de-hairing agent in leather and cosmetic industry, and as component of detergents and edible films (Cortezi et al., 2008).

In recent years, there have been many reports on purification of keratinases from microorganisms (Ferrero et al., 1996; Riffel et al., 2007; Riessen and Antranikian, 2001; Zhang et al., 2009). Depending on protease source, keratinolytic enzymes show different activity and substrate specificity. For instance, keratinase from *B. licheniformis* is capable of hydrolyzing bovine serum albumin, collagen and elastin. On the contrary, a thermostable protease synthesized by *Cryzosphorium keratinophilum* is active only towards keratin (Dozie et al., 1994). The use of keratinolytic enzymes from thermohiles leads to increased rate of keratin degradation. Moreover, such keratinases usually have low collagenolytic activity, which are the requirements of enzymatic de-hairing in the leather industry. The conventional method of de-hairing in an alkaline condition by treatment with sodium sulfide create problems of environmental pollution (Gupta and Ramnani, 2006; Macedo et al., 2005).

Properties of keratinases are dependent on microorganism and synthesis of these enzymes can be induced by keratin added to the growth media. Essential for keratinases production are cultivation conditions, such as pH, temperature and media composition. The majority of known keratinases are extracellular or outer-membrane bound endopeptidases belonging to the serine protease family, but aspartic-, cysteine- and metallo-proteases are also found (Suzuki et al., 2006). Keratinases produced by mesophilic bacteria and saprophytic fungi are mostly active at temperatures up to 50°C. However, in a few cases, thermoactive protease which maintain keratinolytic activity at elevated temperatures are produced by some mesophiles (Dozie et al., 1994). Such phenomenon was observed in the case of *B. licheniformis* K-19 production, during cultivation at 37°C; keratinolytic protease perfo-rmed enzymatic activity at temperatures from 30 to 90°C. This protease has highest activity at 60°C and pH 7.5 - 8.0 (Xu et al., 2009). In contrast, protease from thermophilic *Fervidobacterium islandicum* AW-1 shows optimal keratinolytic activity at 100°C and pH 9.0, and has half-life of 90 min at 100°C (Nam et al., 2002).

The biodegradation employing purified keratinase could be replaced by the action of thermophilic microorganisms growing in reaction media or the use of culture filtrates containing the keratinase alone without microorganism cells (Balint et al., 2005; Fredrich and Antranikian, 1996; Gousterova et al., 2005; Wang and Yeh, 2006). This process is efficient enough and usually requires mild conditions and smaller energy input, because microbial decomposition generates heat during cultivation. It reduces the costs of processing and no pathogenic bacteria (mostly mesophiles) can grow at elevated temperature. Recently, the bacterium *Meiothermus ruber* H-328 was used for utilization chicken feathers. Aerobic cultivation of this moderate thermophile at 55°C for 6 days causes almost complete degradation of the feathers into amino acids and oligopetides (Matsui et al., 2009).

The novel possible application of keratinases and some other microbial proteases is their use for degradation of infectious form of prion proteins created through aggregation to abnormal amyloid structure designated as PrP^{Sc} (Johnson, 2005; Priola, 2001). Generation of this form causes bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jacob disease in human. Aggregated prion molecules are strongly resistant to conventional proteases and different methods used for pathogen inactivation, including autoclaving at 121°C (Langeveld et al., 2003). Recent investigations have shown that amyloid form of prion can be degraded by some microbial proteases through the use of denaturing pretreatments, such as pre-heating and treatment with detergents (Tsiroulnikow et al., 2004). Promising source of enzymes degrading PrP^{Sc} without detergents or under non-alkaline conditions are some thermophiles and microorganisms belonging to the genus of *Streptomyces* (Hui et al.,

2004). Furthermore, thermophiles belonging to *Thermoanaerobacter* and *Thermococcus* were found to hydrolyze the thermally denatured amyloid form of prion and could be used for decontamination of animal wastes (Suzuki et al., 2006).

The activity of the membrane-bound proteases from thermophilic *Geobacillus colagenovorans* MO-1 towards collagen shows that their substrate binding-domains could be useful in enhancing drug delivery in some tissues. For instance, a fusion protein carrying the epidermal growth factor at the collagen binding-domain, when injected into nude mice, remained around sites of injection up to 10 days, whereas not fused substance was not detectable 24 h after injection (Nishi et al., 1998).

Recently, thermostable protease from *Thermus* sp. is produced for cleanup of DNA before polymerase chain reaction (PCR). The application of thermostable enzyme for this purpose is caused by compatibility with an existing high-temperature process (Bruins et al., 2001).

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